

Transmission of mutans streptococci in mother-child pairs

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Background & objectives: Dental caries is an infectious, transmissible disease. Maternal transfer of mutans streptococci (MS) has been a subject of research. The aim of this study was to evaluate the transmission of MS from mother to children through genetic analysis.

Methods: Thirty mother-child pairs were included and divided into three groups according to the age of the children. Saliva samples were collected and MS colonies from each mother-child pair were isolated. After inoculation and incubation, MS colonies were submitted to amplification technique by polymerase chain reaction (PCR) for identification and arbitrarily primed PCRs (AP-PCRs) to determine various MS genotypes.

Results: From birth to six months of age, 30 per cent of children exhibited MS colonization, and by the age of 30 months, 100 per cent harboured the bacteria ($P < 0.001$). Factors associated with MS colonization were eruption of teeth ($P < 0.001$), feeding habits with mean colony count being significantly lower in breast-fed as compared to bottle-fed children ($P < 0.001$) and a significant association between mean MS count of child and mother's practice of sharing spoon with child ($P < 0.001$). The AP-PCR fingerprinting profile analysis showed 17 MS groups (clusters) containing identical or highly related isolates in mother-child pairs with a high level of similarity (77.27 %).

Interpretation & conclusions: The presence of matching MS genotypes suggested vertical transmission from mothers to children. Feeding habits, gum cleaning and number of erupted teeth in children had significant effect on MS colonization. There is a need to develop strategies to prevent MS colonization in children.

Key words Acquisition - colonization - DNA fingerprinting - fidelity - mother-child pairs - mutans streptococci

Dental caries is an infectious and transmissible disease. The tooth-associated biofilm comprises mainly mutans streptococci (MS), which have cariogenic activity under acidogenic conditions. Coexistence with other microbial populations harbours the ideal niche for caries development. MS and a few lactobacilli species are known to be infectious agents strongly associated with dental caries¹. Early colonization of

MS is related to high caries activity during childhood¹. Earlier studies demonstrated that infants acquire MS from their mothers only after the eruption of primary teeth². Children acquire MS during a discrete period between the age of 19 and 33 months, designated as the 'first window of infectivity', and the source of initial infection mostly is through mother². It has been demonstrated that MS can colonize the mouths of

pre-dentate infants and horizontal as well as vertical transmission occurs^{3,4}. Earlier studies demonstrated fidelity of maternal transfer to be as high as 71 per cent in a cohort of Birmingham children⁵ and as low as 43 per cent in Toronto, Ontario, children⁶. Emanuelsson *et al*⁷ reported 55 per cent of maternal transmission and no paternal transmission in Swedish families.

It is important to determine MS source in infants to assess infectivity. Hence, this study was aimed to assess the colonization of MS in children (from birth to 30 months) and to assess whether a correlation exists between the magnitude of maternal reservoirs of MS and the likelihood of transmitting the infection to children by microbial and molecular (PCR) assay.

Material & Methods

The study was conducted in MM College of Dental Sciences and Research, Mullana (Haryana), India, in collaboration with MM Medical College and MM College of Bio Technology from 2009 to 2011. Mother-child pairs were selected from the medical college and hospital outpatient department, Neonatal department and from the nearby *Anganwadi* in Mullana and Barara suburbs. The objective of the study was explained in detail to the respective mothers in their local language. The participation in the study was voluntary, and informed written consent was obtained. The study protocol was approved by the ethics committee. A total of 120 mother-child pairs were screened. Thirty mother-child pairs were selected who willingly participated and fulfilled the inclusion criteria.

The selection criteria were (i) neither the mothers nor the pre-school children (from birth to 30 months of age) were under medication for the past six weeks, and (ii) none had any history of major illness or hospitalization. The pre-school children with natal or neonatal teeth and mothers undergoing orthodontic or prosthodontic treatment were excluded.

Selected mother-child pairs were categorized into three groups: (i) Group I: absence of erupted teeth in children during the collection visit, pre-dentate (10 children; age – birth-6 months) and their mothers; (ii) Group II: presence of 1-5 primary teeth (excluding molars) (10 children; age – 7-18 months) and their mothers; and (iii) Group III: presence of 6-8 primary teeth (including molars) (10 children; age – 19-30 months) and their mothers.

The caries status of mothers was recorded as per the WHO 2004 criteria⁸.

Saliva samples of the mother-child pairs were collected using a sterile cotton swab, kept below the ventral surface of the tongue until saturated and then transferred immediately to duly labelled screw-capped sterile polypropylene tubes. The saliva samples were dispersed from swabs and diluted in Todd Hewitt broth (HiMedia, Mumbai).

Mitis salivarius-bacitracin (MSB) agar (HiMedia, Mumbai) was prepared by adding 0.2 units of bacitracin/ml and sucrose to 20 per cent concentration of mitis salivarius agar. Aliquots of saliva samples were inoculated on plates containing mitis salivarius-bacitracin agar. The plates were incubated at 37°C for 48 h in an atmosphere of five per cent CO₂ in the candle jar. Smears on plain slides were prepared, Gram staining was performed and stained sections were examined using Nikon research microscope (ECLIPSE 80i, NY, USA) with CCD video camera (NIKON DS.U2, 5.03, NY, USA) at ×100 resolution under oil immersion.

The colonies were identified by their morphology, *i.e.* round, spherical, raised, blue ranging from pinpoint to pinhead size with rough surface and detachable from the agar surface. A few colonies from the cultures were randomly selected and biochemically tested using mannitol fermentation, oxidase and catalase tests. The colony counts of the mother and child in colony-forming unit (cfu)/ml were counted using colony counter.

DNA isolation: The procedure of DNA isolation, digestion and electrophoresis and gel analysis was done as per the method described by Bert *et al*⁹. Culture colonies of typical morphotypes from children and mothers were collected, seeded and grown overnight at 37°C (5% CO₂) in 20 ml brain–heart infusion broth. An aliquot (2 ml) of bacterial culture was submitted to bacterial identification. The bacterial identification of MS was performed by PCR.

DNA was extracted using a simple DNA preparation. The cells were washed twice in TE buffer (1 M Tris-Cl, pH 8.0, 0.5 M EDTA, pH 8.0), centrifuged and re-suspended in TE buffer. DNA was released from bacterial cells by incubation with 30 µl of 10 per cent sodium dodecyl sulphate and 10 mg/ml lysozyme for 30 min at 60°C, followed by incubation with 10 mg/ml

proteinase K for one hour at 65°C. After this, 100 µl of 5 M NaCl and 80 µl of cetyltrimethylammonium bromide (CTAB)/NaCl were added and mixed thoroughly and incubated at 65°C for 10 min. The pellets were treated several times with phenol:chloroform:isoamyl alcohol (25:24:1v/v), and finally, DNA was precipitated with cold ethanol. DNA was dried, suspended in TE buffer and stored at -20°C. The same procedure was followed for all the cultures.

PCR identification and arbitrarily primed (AP)-PCR typing: The DNA samples from MS isolates were identified by PCR with primers: *Streptococcus mutans* (*gtfB*, glucosyltransferase β gene) primer pairs F, 5'-ACT ACA CTT TCG GGT GGC TTG G-3' and R, 5'-CAG TAT AAG CGC CAG TTT CAT C-3' (517 bp) (GeneX India Bioscience Pvt. Ltd., Chennai, India). The PCR was processed in 25 µl of a reaction mixture containing ×1 reaction buffer Taq polymerase (2.5 µl), 1.5 mM MgCl₂ (1.5 µl), 0.1 mM deoxynucleoside triphosphate (dNTPs) (0.6 µl), 0.2 µM each primer (0.5 µl), 500 U of Taq DNA polymerase (0.6 µl) and 5 µl of DNA sample¹⁰. PCR amplification was performed with a PCR Thermocycler (Applied Biosystems, 5720 PCR system, USA) under thermal conditions. The reaction mixture was denatured at 95°C for five min, followed by a series of amplification-denaturation steps at 95°C for 30 sec, annealing at 59°C for 30 sec and extension at 72°C for one min, which was repeated for 30 cycles, with a final extension at 72°C for seven min. The PCR amplification products were separated by electrophoresis on 0.8 per cent agarose gel with tris-acetate-EDTA running buffer (pH 8.0).

Isolates identified as MS by PCR method were used for genotyping. The AP-PCR fingerprinting was performed with primer OPA-13 (5'-CAGCACCCAC-3') (GeneX India Bioscience Pvt. Ltd., Chennai, India). AP-PCR¹⁰ was performed in 25 µl mixtures containing 2.5 µl of ×1 reaction buffer, 7 mM MgCl₂, 0.2 mM dNTPs, 0.4 mM primers, 5 U Taq DNA polymerase and 5 µl DNA sample. The samples were placed in Applied Biosystems, 5720 Thermocycler using the following conditions: 45 cycles of denaturation at 94°C for 30 sec, annealing at 36°C for 30 sec and 72°C for one min, with an initial denaturation at 94°C for five min and a final extension at 72°C for five min. Amplification products were analysed electrophoretically, with 0.8 per cent agarose gel with tris-acetate-EDTA running buffer (pH 8.0).

Analysis of the gels: The 100 bp DNA ladder (BIORON, Germany) was used as a molecular marker. Each gel had 13 wells. The molecular marker was run in the first well, and the DNA isolates of each of the six mother-child pairs were run in the remaining 12 wells. Thus, each gel showed the band patterns of six mother-child pairs. The band patterns obtained on the gels were viewed on an ultraviolet viewer, and the gels were photographed using a digital camera. The band patterns for each of the individuals, *i.e.* the mother and child, were compared with the standard (molecular marker) for the similarity in appearance.

Statistical analysis: The data were analyzed using ANOVA, and the F - test was used to compare the differences among the groups. When significant differences were detected, pairwise comparisons were made between all the groups using Tukey's method to adjust for multiple comparisons.

Results

The mean age of children in Group I was 2.60 ± 1.84 months; that in group II was 9.50 ± 0.53 months; and in group III 23.60 ± 3.57 months. Three children (30%) of group I, 9 children (90%) of group II, 10 children (100%) of group III and mothers of all included children showed growth of MS.

The mean colony counts of MS among children were seen to be increasing significantly with increasing age. It was observed that mean colony count in group I was 0.28 ± 0.48 × 10² cfu/ml whereas the same were observed to be 2.92 ± 2.20 × 10² and 4.76 ± 3.06 × 10² cfu/ml, respectively, in groups II and III (*P* < 0.001). The mean MS count of mothers in group I was 2.39 ± 1.51 × 10⁵ cfu/ml whereas the same were 2.36 ± 1.95 × 10⁵ and 1.83 ± 0.85 × 10⁵ cfu/ml in groups II and III, respectively.

No significant association between mode of delivery and mean MS count of pre-school children was observed. However, a significant correlation between mean number of erupted teeth and mean MS count was observed in pre-school children (*ρ* = 0.755; *P* < 0.001). There was a significant association between MS count and feeding habits with the mean count being significantly lower among those who were breast-fed as compared to those who were bottle-fed (*P* < 0.001). Gum cleaning habit in pre-school children showed a significant association with MS colony count (*P* < 0.05). No significant association between habit of mother kissing children on mouth and MS count was observed

though the mean count was significantly higher among those observing this practice as against those who did not. A significant association between mean MS count of children and mother's practice of sharing spoon with child was observed ($P < 0.001$). A similar association was observed with practice of family members sharing food with child (Table).

The mother-child pair numbers 1, 5, 13, 18, 21, 22, 25 and 30 who failed to show any growth were therefore, excluded from the process of DNA isolation and for PCR analysis. Three children of group I, six of group II, 10 children of group III and three mothers of group I, eight mothers of group II, nine mothers group III showed positive results. Two children and one mother of group II and one of mother of group III did not show positive results.

Dendrogram analysis of AP-PCR fingerprinting profile with primer OPA-13 and molecular marker 100 bp DNA ladder showed distinct genotypes' patterns

of MS obtained from mother-child saliva samples (Fig. 1).

The same genotypic pattern was considered for identical or related samples with genetic similarity with slipped-strand mispairing (SSM) ≥ 0.65 (threshold). The genetic similarity values ranged from $0.07 \leq \text{SSM} \leq 1$. Based on the matrices generated by the principal component analysis (PCA) using coefficient SSM, the genetic similarity levels among the MS isolates were obtained. Fig. 2 shows representative dendrograms of MS isolates obtained for each mother-child pair (22 pairs). Seventeen MS groups (clusters) containing identical or highly related isolates (SSM ≥ 0.65) were established in mother-child pairs. A high level of similarity (77.27%) occurred between children and mother.

Discussion

The mother is considered to be the important source of transmission of infection in children due to intimate

Table. Association of maternal, birth, hygiene and habit-related variables with mutans streptococci colony count

Variable	Number of cases	Mean cfu $\times 10^2/\text{ml}$	SD	Median cfu $\times 10^2/\text{ml}$	Significance of association (Mann-Whitney U-test)
Mode of delivery					
Normal vaginal	22	3.65	3.95	3.1	$P=0.945$
Caesarean section	8	2.29	2.31	2	
Teeth eruption					
Yes	20	3.84	2.76	4.3	$P=0.003$
No	10	0.28	0.48	0	
Feeding habits					
Breast-feed	12	0.35	0.55	0	$P<0.001$ (Kruskal-Wallis test)
Bottle-feed	8	3.56	2.09	3.2	
Mixed diet & breast-feed + bottle-feed	10	3.98	3.23	2.34	
Gum cleaning habit					
Yes	19	1.86	2.70	1.3	$P=0.013$
No	11	4.01	2.60	3.9	
Mother kissing baby on mouth					
Yes	22	3.22	2.97	3.1	$P=0.067$
No	8	1.09	1.69	0.7	
Mother sharing spoon with child					
Yes	17	4.29	2.72	3.9	$P<0.001$
No	13	0.50	0.76	0.7	
Child sharing food with other family members					
Yes	15	4.42	2.86	3.8	$P<0.001$
No	15	0.89	1.30	0.8	

SD, standard deviation; cfu, colony-forming unit

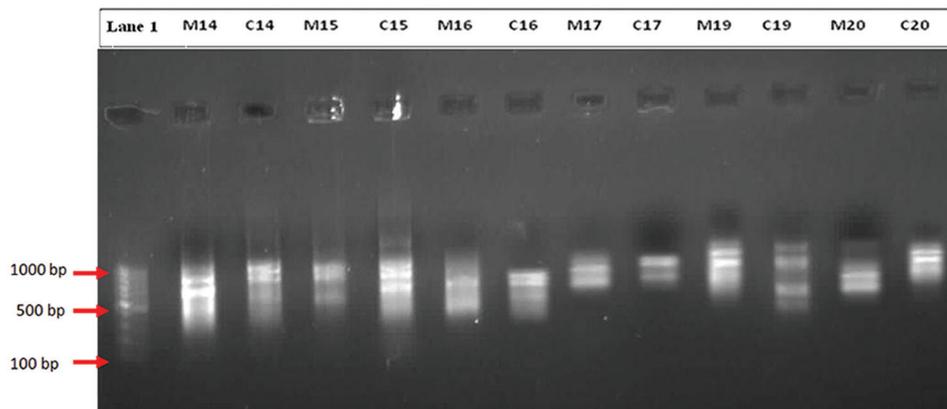


Fig. 1. Genetic similarity levels obtained among the mutans streptococci strains for mother-child pair by arbitrarily primed polymerase chain reaction. Lane 1 represents 100 bp molecular weight marker. M lanes represent mother while C lanes represent child in respective pairs.

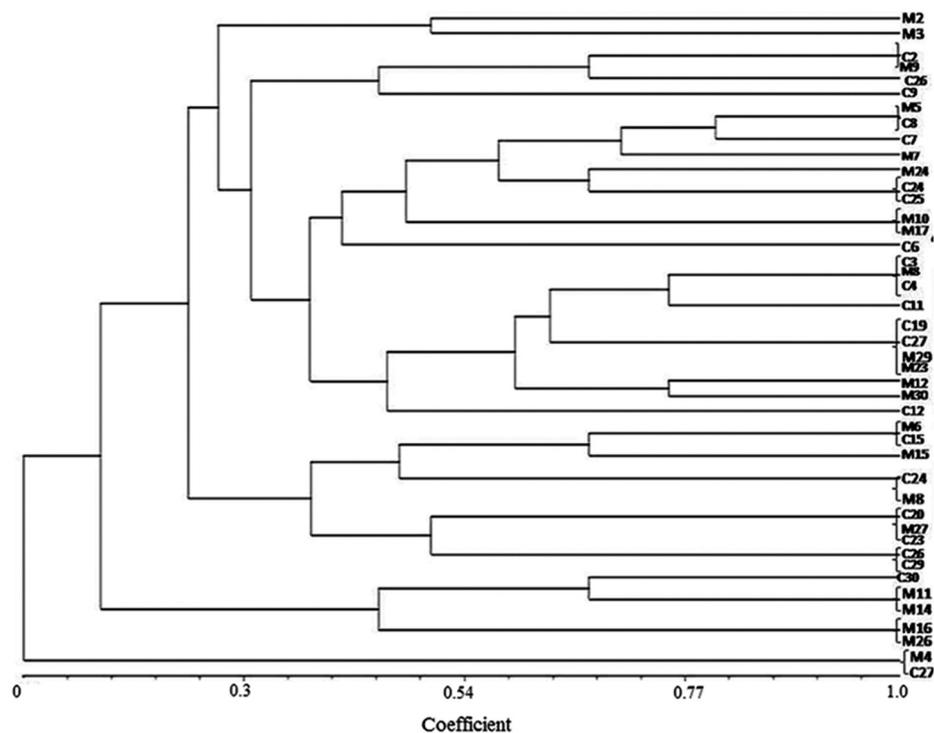


Fig. 2. Dendrograms of mutans streptococci isolates obtained for each mother-child pair (22 pairs) generated by the principal component analysis using coefficient slipped-strand mispairing.

contact with their children in the first two years of life when MS are initially transferred¹. The first indication of this was obtained using a phenotypic technique of MS, *i.e.* bacteriocin typing¹¹. The first tooth erupts at the age of eight months (± 2 months), and only after eruption, there can be stable colonization of MS¹². Pivotal to determining the source of MS in children is the method for identifying individual strains of MS and confirming that the strains found in the mother are the same as those recorded in her child^{5,13,14}.

In the present study, MS was detected in 22 of the 30 children (77.3 %). In group I, 30 per cent children showed growth of MS which was supported by an earlier study which confirmed more than 30 per cent of pre-dentate children infected at the age of three months with MS and over 60 per cent showed the presence of the bacteria by the age of six months¹⁵. In children aged 7-18 and 19-30 months, 90 and 100 per cent, respectively showed MS growth. Karn *et al*¹⁶ reported 27 per cent MS infection in children aged 12 months,

Milgrom *et al*¹⁵ reported MS in 53 per cent of children aged 6-12 months and 72 per cent in those aged 13-24 months and Thorild *et al*¹⁷ detected MS in 30 per cent of 18 month old children.

The MS count in children increased with increasing age as has been reported earlier¹⁸. Child-nurturing habits which facilitate saliva transfer from adults to the children, such as sharing of food and utensils, and habits which involve close contact, such as breast-feeding, kissing and sleeping beside the mother, were also found significantly associated with colonization of MS^{19,20}. Berkowitz *et al*²¹ have reported that mothers with salivary levels of MS >10⁶ organisms per millilitre of saliva have >50 per cent rate of transmission of the bacteria to their 10-16 months old children compared with a rate of only 30 per cent in the case of mothers with only 10³ organisms per millilitre of saliva.

In the present study, the correlations between the acquisition of MS with the mode of delivery, infant's feeding habit, gum cleaning of infant, number of erupted teeth in pre-school children, maternal caries status and maternal habits were evaluated. A previous study has hypothesized that the caesarean delivery accelerates initial acquisition of MS due to less exposure to the maternal microbiota at birth²². The vaginally delivered new-borns come into contact with greater numbers and varieties of bacteria from the perineum (vagina and anus) earlier and with greater intensity than do the relatively aseptically delivered caesarean-born babies. Feeding habit, gum cleaning and sharing of spoon with mother and family members confirmed significant influence on MS colonization in the children as has been reported earlier also²³. Thus, mothers and other family members who share food, drinks, utensils, toothbrushes and other items with their children have the highest risk of transmitting MS to their children²⁴.

In the present study, eight mother-child pairs who failed to show any growth on MSB agar, were excluded from the process of DNA isolation and for PCR and AP-PCR analysis. The results for the remaining 22 mother-child pairs revealed that MS identification by both MSB and PCR did not show significant differences. However, four samples (two pre-school children and one mother in group II and one mother in group III) who were positive in MSB showed a negative result for PCR.

The presence of matching genotypes of MS was similar in 77.27 per cent (17 of 22) mother-child pairs, suggesting vertical transmission. Klein *et al*²⁵ detected

81.25 per cent genotypic similarity between mother and child using AP-PCR fingerprinting profile analysis. Li *et al*²² registered 71 per cent similarity between the bacterial strains using the DNA fingerprinting technique; Grönroos *et al*²⁶ observed 64 per cent genetic similarity in mother-child pairs from Finland (using the immunodiffusion, ribotyped and mutacin-typed techniques).

In conclusion, MS may be acquired through both vertical and horizontal transmissions. Mothers are the main source of infection to their children. However, alternative paths for *S. mutans* transmission have also been reported²⁷. The present findings showed increasing mean colony counts among children with increasing age. Mode of delivery and habit of kissing by mother did not show significant influence on MS colonization in the children whereas feeding habit, gum cleaning and number of erupted teeth in children and sharing of the spoon with mother and family members showed significant influence. The presence of matching genotypes of MS by PCR and AP-PCR suggested vertical transmission among mother-child pairs. These findings will facilitate the development of clinical strategies to prevent or delay infant infections by various organisms.

Conflicts of Interest: None.

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